Apical Sensory Organ in Larvae of the Patellogastropod *Tectura scutum*

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Abstract. The apical sensory organ in veliger larvae of a patellogastropod, a basal clade of gastropod molluscs, was studied using ultrastructural and immunohistochemical techniques. Immediately before veligers of *Tectura scutum* undergo ontogenetic torsion, the apical sensory organ consists of three large cells that generate a very long apical ciliary tuft, two cells that generate a bilateral pair of shorter ciliary tufts, and a neural ganglion (apical ganglion). Putative sensory neurons forming the ganglion give rise to dendrites that extend to the apical surface of the larva and to basal neurites that contribute to a neuropil. The ganglion includes only one ampullary neuron, a distinctive neuronal type found in the apical ganglion of other gastropod veligers. Serotonin immunoreactivity is expressed by a medial and two lateral neurons, all having an apical dendrite, and also by neurites within the neuropil and by peripheral neurites that run beneath the ciliated prototrochal cells that power larval swimming. The three cells generating the long apical ciliary tuft are lost soon after ontogenetic torsion, and the medial serotonergic cell stops expressing serotonin antigenicity in late-stage veligers. The lateral ciliary tuft cells of *T. scutum* may be homologs of lateral ciliary tuft cells in planktotrophic opisthobranch veligers. A tripartite arrangement of sensory dendrites, as described previously for veligers of other gastropod clades, can be recognized in *T. scutum* after loss of the apical ciliary tuft cells.

Introduction

The apical sensory organ of larval gastropods has potential value for studies on patterns and mechanisms of evolutionary change during development. Existing evidence, albeit fragmentary, suggests that at least some cellular

components of the apical sensory organ in veliger larvae of gastropods and in larvae of other molluscs are segregated early in embryogenesis and have a similar pattern of embryonic cell lineage (see Raven, 1966; Camey and Verdonk, 1970; Cather, 1973; van Dongen and Geilenkirchen, 1974; Verdonk and van den Biggelaar, 1983; Dohmen, 1992; Dictus and Damen, 1997). Studies on planktotrophic gastropod veligers have shown that the apical sensory organ is a discrete morphological unit consisting of a small neural ganglion (apical ganglion) and, in most species examined to date, a bilateral pair of ciliary tufts (Kempf *et al.*, 1997; Marois and Carew, 1997a; Page and Parries, 2000). These morphological data suggest that the apical ganglion includes sensory neurons and peripheral neurites extending from the ganglion innervate muscles and prototrochal cilia of the velum. Furthermore, recent experimental research on veligers of the nudibranch gastropod *Phestilla sibogae* has justified the long-held belief that the apical sensory organ detects the exogenous cue for induction of larval metamorphosis (Hadfield *et al.*, 2000).

Although the larval apical sensory organ of gastropods should be amenable to investigations of the developmental constraints and novelties that have interacted to produce evolutionary differences in final morphology, the solid platform of comparative data that such studies require is currently incomplete. To date, detailed morphological information is available on the apical sensory organ of planktotrophic veligers of caenogastropods (Uthe, 1995; Page and Parries, 2000) and heterobranchs (Bonar, 1978; Chia and Koss, 1984; Kempf *et al.*, 1997; Marois and Carew, 1997a, b, c). However, these two sister groups constitute the most derived clade of extant gastropods, the Apogastropoda (Haszprunar, 1988; Ponder and Lindberg, 1997). Very little is known about the morphology of the apical sensory organ in basal clades of gastropods. There-

fore, we cannot know which, if any, features of the apical sensory organ in larval apogastropods are broadly conserved throughout this molluscan class, much less speculate about the polarity of evolutionary change in the case of differences or the nature of modified developmental mechanisms.

The apical sensory organ of larval molluscs (see Kempf *et al.*, 1997, for a survey of other terms applied to this structure) may have homologs within the larval body plans of other spiralian phyla (Nielsen, 1995). Pioneering ultrastructural work by Lacalli (1981, 1984) showed that the apical organ of *Spirobranchus* trochophores (Polychaeta) is a structurally integrated unit consisting of neuronal cells in addition to cells generating a single long tuft of cilia. Neurites extending from the neurons intermingle within a plexus or neuropil so that a simple type of ganglion is formed. In veliger larvae of derived gastropod groups, the ganglionic component of the apical sensory organ consists of sensory and nonsensory neurons clustered over a neuropil (Kempf *et al.*, 1997; Marois and Carew, 1997a; Page and Parries, 2000). Dendrites extending from the sensory neurons are organized into three bundles (a medial and two lateral bundles) that penetrate the overlying epithelium (see also Chia and Koss, 1984). A subgroup of the sensory neurons, called ampullary neurons, have a deep invagination of the peripheral, dendritic membrane that is filled with cilia arising from the wall of the invagination. In addition, antibodies against serotonin label (1) three to six neuronal somata within the apical ganglion (the number is constant within each species that has been studied), (2) neurites within the neuropil of this ganglion, and (3) peripheral neurites that extend from the neuropil to muscle fibers and prototrochal ciliated cells of the larval velum (Kempf *et al.*, 1997; Marois and Carew, 1997a; Page and Parries, 2000).

A major enigma regarding the comparative structure of the apical sensory organ among gastropod larvae relates to the distinctive long ciliary tuft that is a characteristic component of this larval structure in many other spiralians, including at least some members of the molluscan classes: Scaphopoda, Polyplacophora, Aplacophora, and Bivalvia (see reviews by Raven, 1966; Verdonk and van den Biggelaar, 1983). Among gastropods, a long cohesive tuft of non-vibratile cilia arising from the center of the larval apical epidermis has been reported only for members of the Patellogastropoda (Smith, 1935; Dictus and Damen, 1997; Wanninger *et al.*, 1999, 2000), which is currently viewed as the most basal clade of extant gastropods (Haszprunar, 1988; Ponder and Lindberg, 1997). The homolog of the patellogastropod apical tuft in other gastropod groups is unclear. Some authors (Conklin, 1897; Cather, 1973) have suggested that the apical tuft in caenogastropods is represented in prehatching embryos by four apical cells bearing short, motile cilia. However, the fate of these cells in hatching larvae was not determined. More recently, ultrastructural studies on posthatching planktotrophic veligers of both caenogastropods and heterobranchs identified a potential homolog of the patellogastropod apical tuft in the form of two relatively short ciliary tufts that flank the midline at the ventral margin of the apical sensory organ (Chia and Koss, 1984; Kempf *et al.*, 1997; Page and Parries, 2000). As yet, the embryonic derivation of the cells generating these bilateral ciliary tufts in posthatching veliger larvae of caenogastropods and heterobranchs has not been determined.

I investigated the ultrastructure and serotonin immunoreactivity of the apical sensory organ in the patellogastropod *Tectura scutum* (Rathke 1833) with the goal of identifying similarities and differences relative to the apical sensory organ of planktotrophic apogastropod larvae. Overall development of posthatching larvae of *T. scutum* is similar to that described previously for two other patellogastropod species belonging to the genus *Patella* (Smith, 1935; Wanninger *et al.*, 1999, 2000).

Materials and Methods

Source of adults, fertilization of gametes, and culture of embryos and larvae

Adults of *Tectura scutum* were collected during late August and early September from the intertidal zone of rocky shores along the southwestern perimeter of Vancouver Island, Pacific coast of Canada. Adults were isolated into separate bowls of seawater at 12 °C, and 10% to 25% of these animals spawned eggs or sperm beginning at $15\frac{1}{2}$ to 16 h after the time of lower low tide. Freshly spawned eggs were transferred to 1 liter of filtered (0.45 μm) seawater (FSW) so as to cover the bottom of the beaker with a sparse monolayer of eggs. One milliliter of concentrated sperm was mixed into 500 ml of FSW, and 1 to 2 ml of this sperm suspension was added to the eggs and stirred gently for ½ h. Seawater over settled eggs was decanted and replaced with fresh FSW. About 100 fertilized eggs were transferred into culture beakers containing 500 ml FSW at 12 °C. Culture water was changed daily by gently pouring cultures through a 49-μm sieve immersed in a small bowl of seawater. The sieve was constructed by replacing the bottom of a small plastic beaker with Nitex cloth attached by silicon glue. Larvae retained by the sieve were then pipetted into beakers of fresh FSW. To obtain normal development without adding antibiotics, it was important to culture embryos and larvae at very low density. Adult limpets were subsequently returned to the site of collection. Results reported here are based on five batches of larvae reared over three separate years.

Tissue preparation for scanning and transmission electron microscopy (SEM and TEM)

Larvae were pipetted into 8-ml vials and anesthetized by initial incubation for 2 h at 12°C in several changes of

artificial seawater having augmented Mg^{++} and reduced Ca^{++} concentrations (225 mM NaCl, 5 mM KCl, 102 mM MgCl$_2$ 1 mM CaCl$_2$ in distilled water). The volume of fluid was then reduced to 1.5 ml, and a small piece of menthol crystal (7–10 mg) was floated on the fluid surface. After 45 min, the menthol and artificial seawater was replaced with two changes of primary fixative at room temperature.

Primary fixative consisted of 2.5% glutaraldehyde in 0.2 M Millonig's phosphate buffer (pH 7.6) and 0.14 M sodium chloride. Larvae were stored in this fixative at 8° to 10 °C for several days to one month before further processing. Subsequent processing steps were done at room temperature. Larval shells were decalcified in a 1:1 mixture of primary fixative and 10% ethylenediaminotetraacetic acid (disodium salt) for 1 h (Bonar and Hadfield, 1974). Specimens were then rinsed three or four times in 2.5% sodium bicarbonate (pH 7.2) and transferred to a 1:1 mix of 4% OsO$_4$ and 2.5% sodium bicarbonate for 1 h. A graded ethanol series was used to dehydrate fixed specimens, which were then transferred through three changes of propylene oxide and infiltrated and embedded in TAAB 812 epoxy resin (Marivac, Halifax, NS, Canada). The foregoing procedures, from anesthesia of the larvae to infiltration with epoxy resin, were done on specimens contained within squat, wide-mouth vials of 8-ml capacity. Specimens within the vials could be viewed with a stereomicroscope placed on the sill of a fume hood so that solutions could be removed from the vials using a bent pipette without accidentally removing larvae.

Thin sections were cut with a diamond knife and lifted onto 150-mesh copper grids. Central portions of each section were teased over openings in the grid mesh using an eyebrow hair mounted on an orange stick. Dried sections were stained in aqueous 2% uranyl acetate for 1.5 h and in 0.2% lead citrate for 6–7 min and were photographed with a Hitachi 7000 transmission electron microscope operated at 50 kV accelerating voltage.

For SEM, larvae were processed as described above except that specimens in 100% alcohol were transferred into modified Beam capsules for critical point drying from liquid carbon dioxide. The bottom of each Beam capsule was removed, and the top and bottom were covered with a small piece of Nitex cloth of 49-μm mesh size. The Nitex was held in place by sandwiching it between the Beam capsule itself and a Beam capsule lid from which a large hole had been cut. Specimens dried at critical point were transferred to double-sided sticky tape on an SEM stub, sputter-coated with gold, and photographed with a Hitachi 6500N scanning electron microscope operated at 8 to 15 kV accelerating voltage.

Immunohistochemistry

Anesthetized larvae were fixed in a solution consisting of 20 ml 8% paraformaldehyde (freshly prepared from powder according to instructions given in Hayat, 1970), 10 ml of 0.2 M Millonig's phosphate buffer (pH 7.6) and 10 ml of 0.14 M sodium chloride (final paraformaldehyde concentration was 4%). Specimens were fixed at 6 °C for 3 h, rinsed six times over an hour in phosphate-buffered saline (PBS) at pH 7.4 containing 0.1% sodium azide, and stored in the final rinse for 12 h to 4 days before further processing. All subsequent incubations were done at 6 °C on an orbital shaker. Fixed larvae were blocked for 4 h in PBS containing 0.1% Triton-X 100, 0.1% sodium azide, and 0.1% heat-inactivated goat serum, then incubated for 24 h in a rabbit polyclonal IgG against 5-hydroxytryptamine (serotonin) conjugated to paraformaldehyde (DiaSorin, Stillwater, MN; formerly Incstar) at a dilution of 1:1000 in blocking medium. This was followed by six to eight rinses in PBS + 0.1% Triton-X 100 + 0.1% sodium azide over 10 h.

To obtain a fluorescent label of primary antibody location, specimens were incubated in goat anti-rabbit secondary antibodies coupled to the fluorphore Alexa 488 (Molecular Probes, Eugene, OR) at a dilution of 1:250 in blocking medium for 24 h. These were then rinsed 6–8 times in PBS + 0.1% sodium azide and examined with a Zeiss Axioskop compound microscope or a Zeiss LSM 410 confocal laser scanning microscope using filter sets appropriate for fluorescein isothiocynate fluorescence.

To obtain a label of primary antibody location for visualization in sections examined by light or transmission electron microscopy, the secondary antibody was a biotinylated goat anti-rabbit IgG (Histostain kit, Zymed, San Fransisco, CA). After rinsing in PBS + 0.1% Triton-X 100 (no azide), specimens were transferred to a conjugate of streptavidin-horseradish peroxidase (Histostain kit) for 12 h. Specimens were then rinsed six to eight times over 10 h in PBS + Triton-X 100, followed by incubation for 30 min in diaminobenzidine (solution provided in kit marketed by DAKO Diagnostics, Mississauga, ON, Canada) diluted into PBS. Specimens were then transferred to diaminobenzidine diluted into the buffered substrate (containing hydrogen peroxide) supplied in the DAKO kit. After 30 to 40 min, the reaction was stopped by rinsing in PBS + 0.1% sodium azide. Specimens were postfixed in a 1:1 mix of 4% OsO$_4$ and 2.5% sodium bicarbonate, processed into epoxy resin, and sectioned for light or transmission electron microscopy as described above. Sections for light microscopy were cut at 1-μm thickness and stained with methylene blue and azure II in borax (Richardson *et al.*, 1960). Sections for TEM were cut at 80 nm and stained for 45 min in aqueous 2% uranyl acetate and 5 min in 0.2% lead citrate.

Images obtained from the transmission electron microscope were recorded on film. Digital images obtained from the scanning and confocal laser scanning microscopes were imported into Adobe Photoshop and adjusted for contrast, brightness, and image sharpness.

My analysis of the ultrastructure and serotonin immuno-

reactivity of the ganglion and associated ciliary structures of the larval apical sensory organ of *T. scutum* concentrated on larvae that were fixed at three stages: 52 h postfertilization (at the onset of ontogenetic torsion), 90 h postfertilization (30 h after completion of ontogenetic torsion), and 7 days postfertilization (onset of metamorphic competence). Several other stages were examined less thoroughly by either electron microscopy or immunohistochemistry.

Results

Overview of larval development and the apical sensory organ

Larvae of *Tectura scutum* hatch from the egg investments beginning at 12 to 15 h postfertilization (12 °C) and swim by means of long cilia of the prototroch, or pre-oral ciliary band. These trochophore-like hatching larvae are converted to the veliger larval form by secretion of the larval shell and swelling of the foot rudiment over the next 1½ days of development (Fig. 1A). Ontogenetic torsion, a morphogenetic movement involving a 180° rotation of the cephalopodium relative to the shell, mantle, and differentiating viscera, occurs between 52 and 58 h postfertilization. The foot enlarges considerably over the following days (Fig. 1B) so that larvae are capable of both swimming and pedal crawling by 7 days posthatching. Many larvae at this age undergo metamorphosis when added to small bowls containing seawater and pebbles collected from rocky shores inhabited by adults. Metamorphosed individuals lack prototrochal ciliated cells and resume shell secretion to begin the distinctive postmetamorphic teleoconch.

As veligers of *T. scutum* approach the onset of ontogenetic torsion, they have an apical sensory organ that consists of two components: neurons organized as a simple ganglion (the apical ganglion) and two types of distinctive ciliary structures. The most obvious ciliary structure in pretorsional veligers is a tuft of very long, nonmotile cilia that arises from the center of the apical epidermis (Fig. 1A, C, D). This long, apical ciliary tuft is flanked by two low papillae (Fig. 1C), each of which is the protruding, multiciliated apex of a single cell (lateral ciliary tuft cells). The centrally located, apical ciliary tuft is lost soon after ontogenetic torsion is completed, and a pair of cephalic tentacles subsequently grow from the apical surface of the veliger (Fig. 1B). Neurons of the apical ganglion give rise to cytoplasmic extensions, which are putative dendrites, that terminate at the exposed apical surface. The sketches in Figure 2 were traced from transmission electron micrographs of sections cut parallel to the apical surface of larvae at 52 and 90 h postfertilization, such as those shown in Figures 3A and 4. The tracings show positional relationships between cells giving rise to the distinctive ciliary structures of the apical sensory organ (the apical and lateral ciliary tuft cells) and six clusters of sensory dendrites arising from neurons of the

apical ganglion. The position of a small pit within the apical epidermis is also indicated in these diagrams, although the pit is obscured by cilia in the scanning electron micrograph shown in Figure 1C. Details of these components are described below.

Ciliary structures of the apical sensory organ

Scanning electron micrographs of veligers at the onset of ontogenetic torsion (52 h post-fertilization) show that the apical surface within the ring of prototrochal ciliated cells is dominated by a field of cilia in the shape of a plus (+) symbol (Fig. 1C). The two axes of the plus are oriented along the dorsal-ventral and left-right axes of the larva, and the apical ciliary tuft arises from the center of the plus-shaped ciliary field (Fig. 1C). Cilia of the apical tuft form a cohesive bundle that is about 80 µm long and extends perpendicular to the planar surface of the apical epidermis (Fig. 1A). The tuft does not show rhythmic undulations and is lost within 4 to 8 h after larvae complete ontogenetic torsion. Many cilia within the left and right arms of the plus-shaped ciliary field extend from the low papillae that are the apices of the lateral ciliary tuft cells (Fig. 1C). Unlike the apical ciliary tuft, the lateral ciliary tufts are retained until larval metamorphosis.

Transmission electron microscopy shows that cilia of the apical tuft arise from three large cells (Figs. 2A, 3A) and are supported by very long and robust ciliary rootlets (Figs. 1D, 3D). These three multiciliated cells also contain longitudinal arrays of microtubules (Fig. 3D), which may provide further structural support for the cells. As shown in Figures 2A and 3A, the apical tuft cells constitute three of the four apical pole cells of pretorsional veligers of *T. scutum*. The fourth cell, which occupies the ventral position for this tetrad, is the ampullary sensory neuron, a distinctive type of apical ganglion neuron that is described below. Loss of the apical ciliary tuft following ontogenetic torsion appears to be due to degeneration of the three apical tuft cells, rather than simple shedding of cilia from cells that remain intact. Figure 4 (asterisk) shows a remnant of an apical tuft cell in a larva fixed at 90 h postfertilization (about 30 h after completing ontogenetic torsion).

Figure 5A shows the protruding apices of the two lateral ciliary tuft cells in a larva fixed at 90 h postfertilization, which is after the three large cells that produce the apical ciliary tuft have disappeared. In younger larvae (immediately prior to ontogenetic torsion), the central group of apical ciliary tuft cells lies between the two lateral tuft cells (Figs. 2A, 3A). Each lateral tuft cell consists of an expanded, multiciliated apex, a narrow middle zone, and a basal perinuclear region that lies at the ventrolateral margins of the apical ganglion on the left and right sides (Fig. 5C). The apparent "shrinkage" of the lateral ciliary tuft cells in the tracing shown in Figure 2B, relative to those shown in
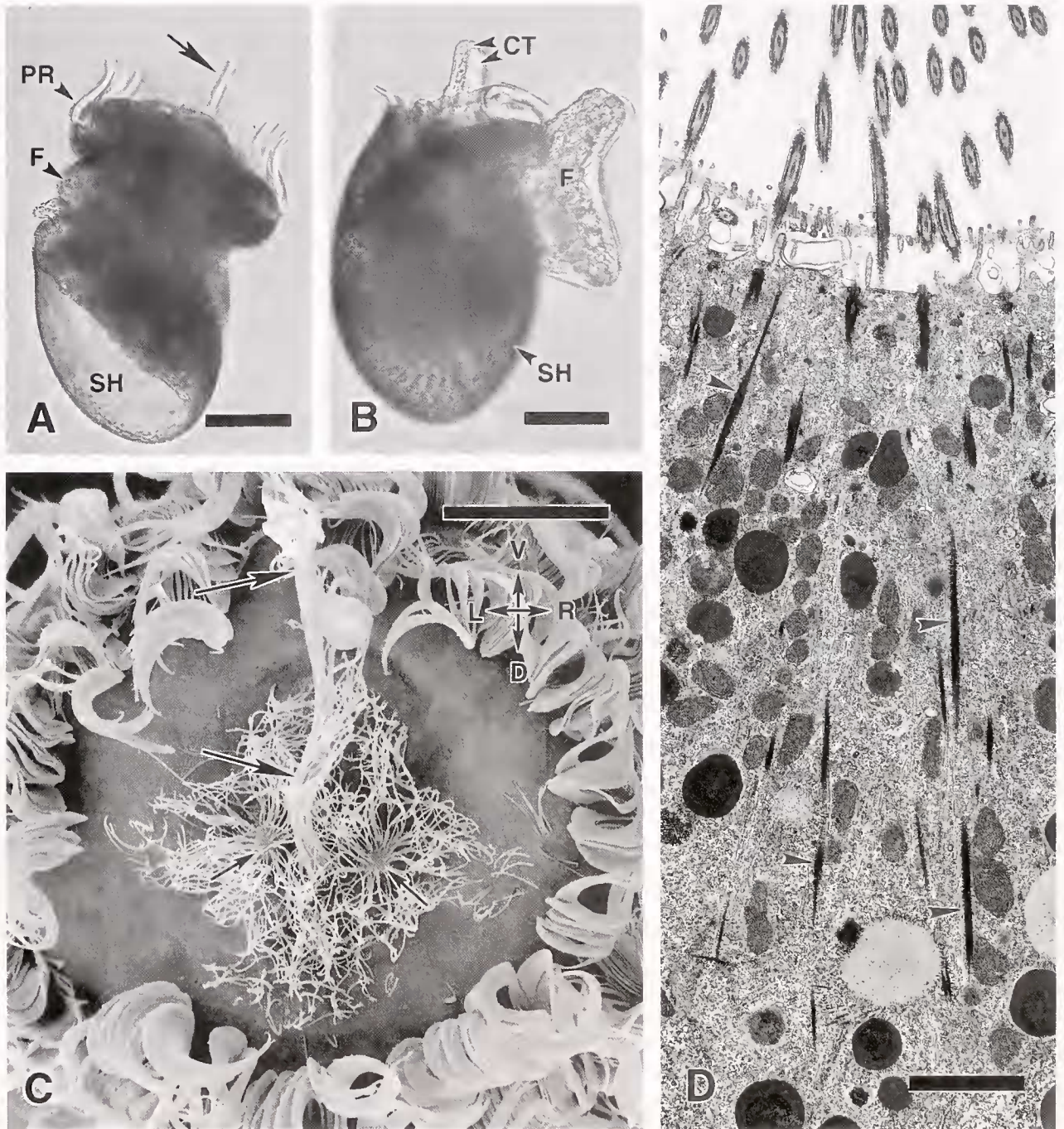
Figure 1. Early and late veliger larvae of *Tectura scutum* showing structures extending from the apical surface. (A) Light micrograph of a live veliger at 46 h postfertilization, prior to ontogenetic torsion; note cilia of the prototroch and the long apical ciliary tuft (arrow). Scale, 50 μm. (B) Light micrograph of a partially retracted veliger at 7 days postfertilization; note the pair of cephalic tentacles and enlarged foot. Scale, 50 μm. (C) Scanning electron micrograph of the apical surface at 52 h postfertilization showing the peripheral ring of prototrochal cilia and the central, plus-shaped ciliary field. The long apical ciliary tuft (large arrows) is flanked by small papillae bearing the lateral ciliary tufts (small arrows). Scale, 20 μm. (D) Transmission electron micrograph showing an apical ciliary tuft cell in longitudinal section from a larva at 48 h postfertilization; note very long ciliary rootlets (arrowheads). Scale, 2 μm. Abbreviations: CT, cephalic tentacles; F, foot; PR, prototroch; SH, shell. Orientation arrows: D, dorsal; L, left; R, right; V, ventral.
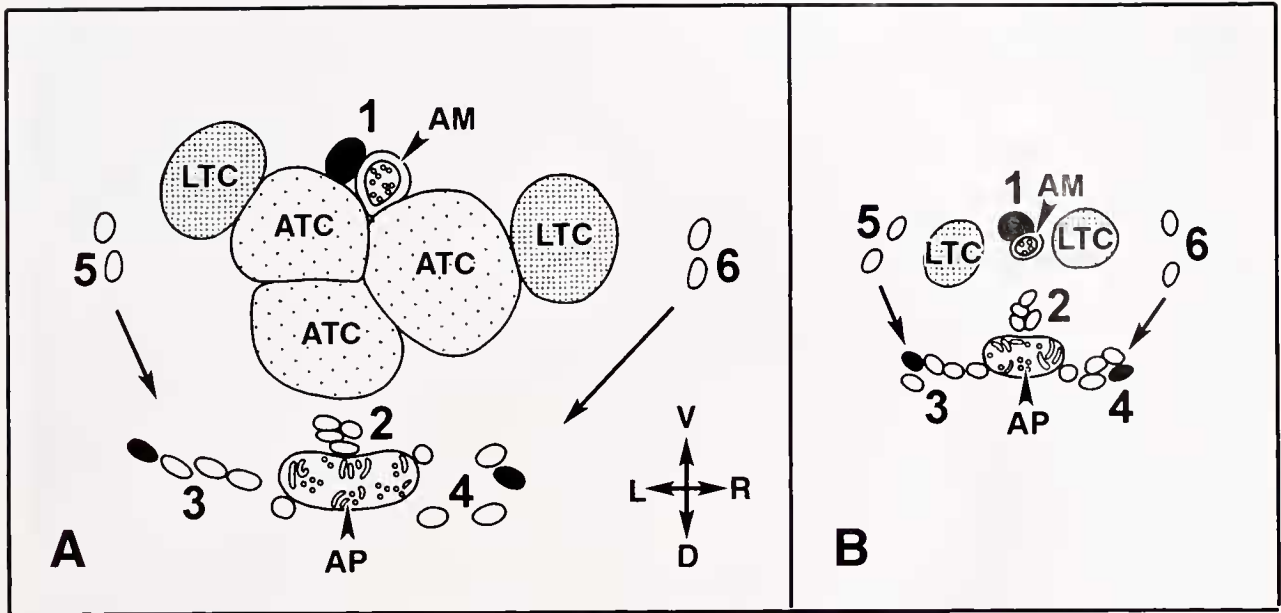
Figure 2. Relative positions of components of the apical sensory organ in veligers of *Tectura scutum* at 52 h postfertilization (A) and 90 h postfertilization (B). Sketches are traced from transmission electron micrographs of sections cut parallel to the apical surface of the larva at a level close to the epithelial surface (same enlargement for both sketches). Numbers refer to groups of dendrites (see text). Arrows indicate that dendrites in groups 5 and 6 join those of groups 3 and 4, respectively, at a deeper level. Filled profiles are dendrites of serotonin-immunoreactive neurons; unfilled profiles are dendrites from other sensory neurons of the apical ganglion. Abbreviations: AM, dendrite of ampullary neuron; AP, apical pit; ATC, apical tuft cell; LTC, lateral tuft cell. Orientation arrows, D, dorsal; L, left; R, right; V, ventral.

Figure 2A, is largely due to the fact that the middle zone of these cells becomes progressively longer and narrower between 52 and 90 h postfertilization. Cilia arising from the lateral ciliary tuft cells are anchored by forked, striated rootlets that are much shorter than the ciliary rootlets extending from the basal bodies of apical tuft cilia.

Apical ganglion

The larval apical ganglion of *T. scutum* is a cluster of neurons located immediately beneath the dorsal half of the apical epidermal disc. Most and possibly all of the apical ganglion neurons give rise to distal cytoplasmic extensions (putative dendrites) that extend to the exposed epidermal surface of the larva within the plus-shaped apical ciliary field. Two to four cilia arise from the distal terminals of these dendrites. The sketch in Figure 2A shows the positions of the dendritic terminals, relative to the apical and lateral ciliary tuft cells and the apical pit, in veligers at the onset of ontogenetic torsion. The sensory dendrites at the exposed surface of the apical epithelium are clustered into six groups: two medial groups and two pairs of lateral groups. Group 1 consists of two dendrites located ventromedially; one of these is the ampullary sensory neuron (Fig. 3A). Group 2 consists of five dendrites that occupy a central location between the large apical tuft cells and the apical pit.

After loss of the three apical tuft cells, dendritic groups 1 and 2 come to lie in close proximity (Fig. 4). Dendritic groups 3 and 4 are located to the left and right, respectively, of the apical pit. Dendritic groups 5 and 6, each consisting of at least two dendrites, are located to the left and right, respectively, of the lateral ciliary tuft cells. After loss of the three large apical tuft cells, the dendrites and the lateral tuft cells shift centrally, but they nevertheless retain the same positional interrelationships (compare Fig. 2A to 2B and Figs. 3A to 4).

The ampullary sensory neuron within dendritic group 1 is a neuronal type that has been identified within the apical ganglion of all other gastropod larvae studied to date (reviewed by Page and Parries, 2000). The distal, dendritic membrane of this neuron is deeply invaginated and gives rise to many cilia that fill the inpocketing (Figs. 3A, 4, 5A). In larvae of *T. scutum*, the invaginated pocket of the ampullary neuron does not penetrate to the level of the basal, perinuclear cytoplasm, and cilia within the pocket have a 9+2 arrangement of axonemal microtubules (Fig. 3C). The ampullary neuron within dendritic group 1 is the only sensory neuron of this type within the larval apical ganglion of *T. scutum*. The second dendrite of the two which comprise dendritic group 1 extends along the left, anterolateral side of the ampullary neuron (Figs. 3A, B, 4). As described below,
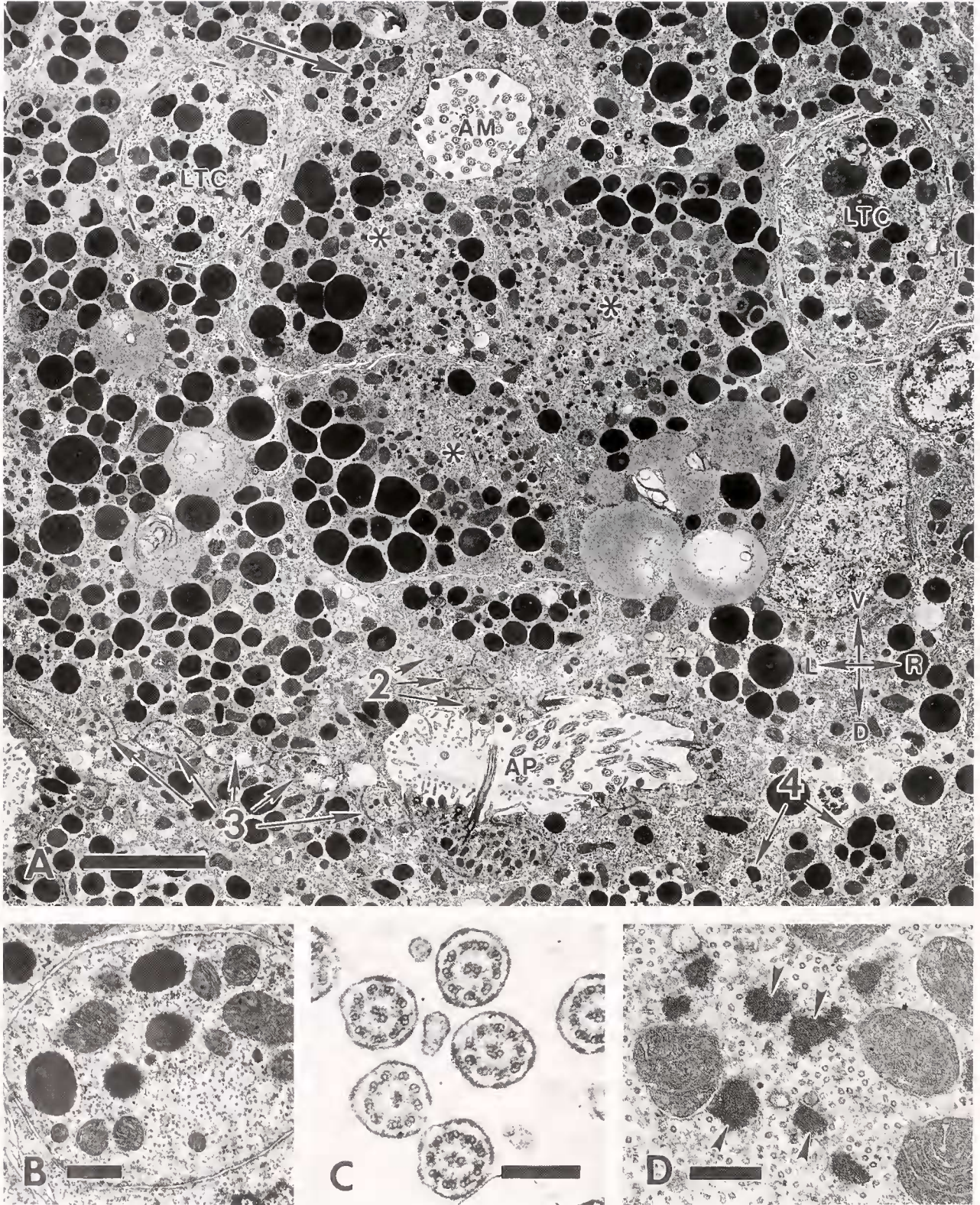
Figure 3. Transmission electron micrographs of a section cut parallel to the apical surface of a *Tectura scutum* veliger at 52 h postfertilization (onset of ontogenetic torsion) showing cellular components of the apical sensory organ. (A) Low-magnification view showing the three large cells (asterisks) that generate the apical ciliary tuft; these are bordered by the apices of lateral ciliary tuft cells (outlined by dashed lines) and dendrites
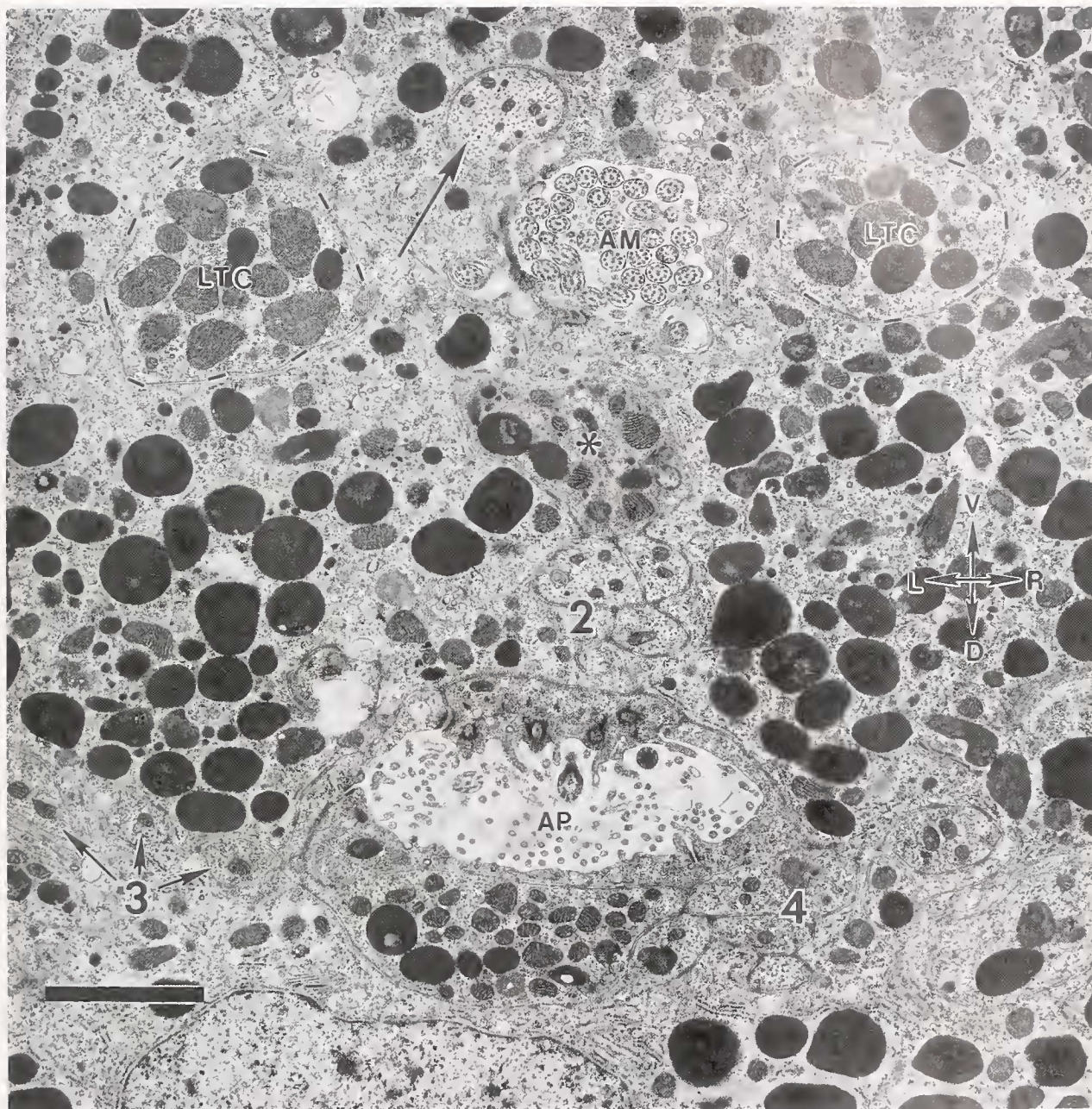
Figure 4. Transmission electron micrograph of a section cut parallel to the apical surface of a *Tectura scutum* veliger at 90 h postfertilization. Note the remnant of an apical tuft cell (asterisk). Arrow indicates dendrite of the non-ampullary neuron of dendritic group 1; dendritic groups 2, 3, and 4 are adjacent to the apical pit; dashed lines outline the lateral ciliary tuft cells. Scale, 2 μm. Abbreviations: AM, ampullary neuron; AP, apical pit; LTC, lateral tuft cells. Orientation arrows: D, dorsal; L, left; R, right; V, ventral.

of the ampullary and non-ampullary (arrow) sensory neurons belonging to dendritic group 1. The section also passes through the apical pit and adjacent dendrites belonging to groups 2, 3, and 4. Scale, 4 μm. (B) Enlargement of the non-ampullary sensory dendrite of dendritic group 1. Scale, 0.5 μm. (C) Detail of cilia within the invaginated pocket of the ampullary neuron; note the 9 + 2 arrangement of axonemal microtubules. Scale, 0.3 μm. (D) Detail of cytoplasm from an apical tuft cell showing robust ciliary rootlets (arrowheads) and small circular profiles of longitudinally aligned microtubules. Scale, 0.3 μm. Abbreviations: AM, ampullary neuron; AP, apical pit; LTC, lateral tuft cells. Orientation arrows: D, dorsal; L, left; R, right; V, ventral.
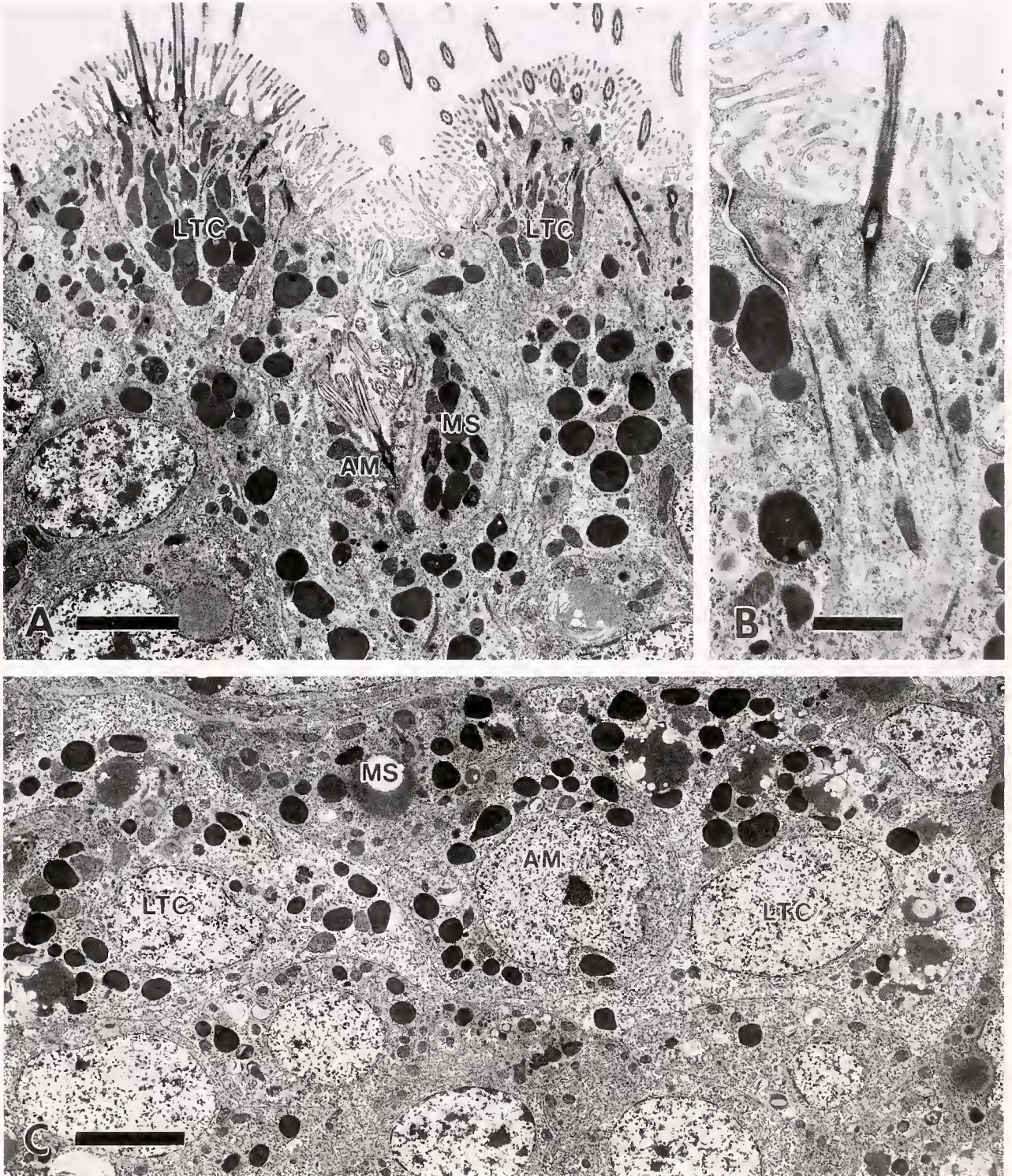
Figure 5. Ciliary and neuronal elements within the ventral portion of the apical sensory organ of *Tectura scutum* at 90 h postfertilization (apical tuft cells are absent at this stage). (A) Transmission electron micrograph (TEM) of a frontal section showing apices of the lateral ciliary tuft cells flanking distal dendrites of the ampullary and non-ampullary neuron (medial serotonergic neuron) of dendritic cluster 1. Scale, 3 μm. (B) TEM of a slightly more ventral section showing a cilium arising from the distal dendrite of the medial serotonergic neuron. Scale, 1 μm. (C) TEM of a section cut parallel to the apical surface at the level of the cell bodies of the apical ganglion. The medial serotonergic neuron, ampullary neuron, and lateral ciliary tuft cells are identified; other neuronal somata of the apical ganglion lie dorsal to these identified cells. Scale, 3 μm. Abbreviations: AM, ampullary neuron; LTC, lateral ciliary tuft cells; MS, medial serotonergic neuron.
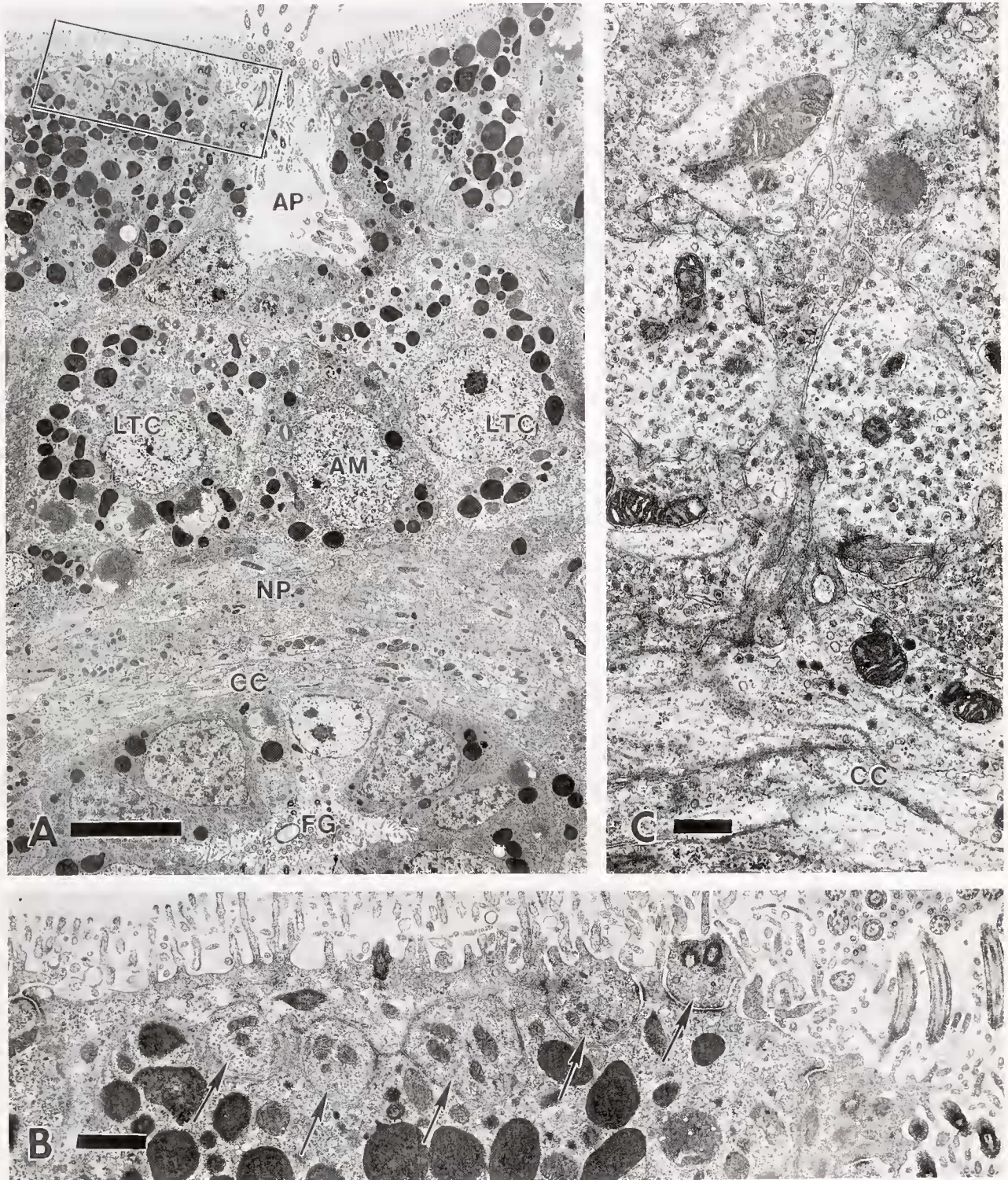
Figure 6. (A) Transmission electron micrograph of a longitudinal section through the apical sensory organ of *Tectura scutum* at 90 h postfertilization showing the apical pit and basal perinuclear regions of the ampullary neuron and lateral tuft cells; neuropil of the apical ganglion overlies the cerebral commissure. Boxed area is enlarged in (B). Scale, 5 µm. (B) Profiles of distal dendrites (arrows) located on one side of the apical pit. Scale, 1 µm. (C) Detail of sectioned neurites within the neuropil of the apical ganglion showing large swellings filled with electron-dense vesicles; a portion of the cerebral commissure is also shown. Scale, 0.5 µm. Abbreviations: AM, ampullary neuron; AP, apical pit; CC, cerebral commissure; FG, foregut; LTC, lateral tuft cells; NP, neuropil.
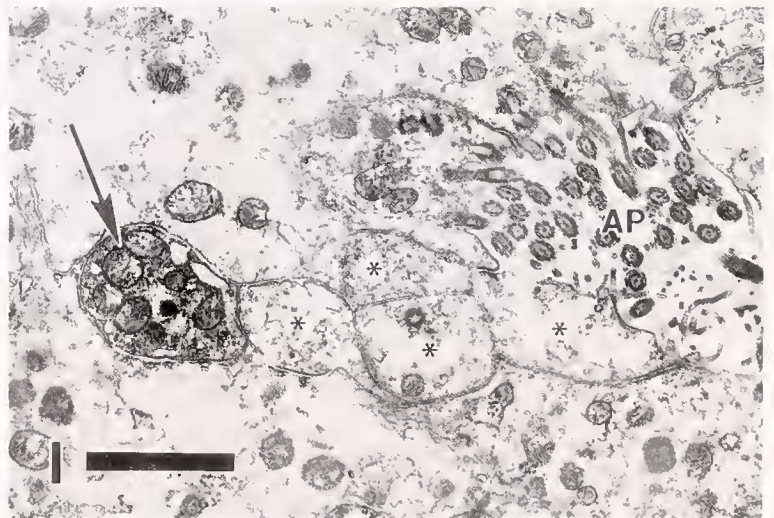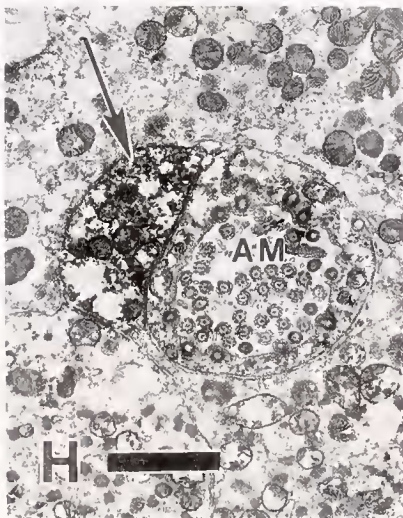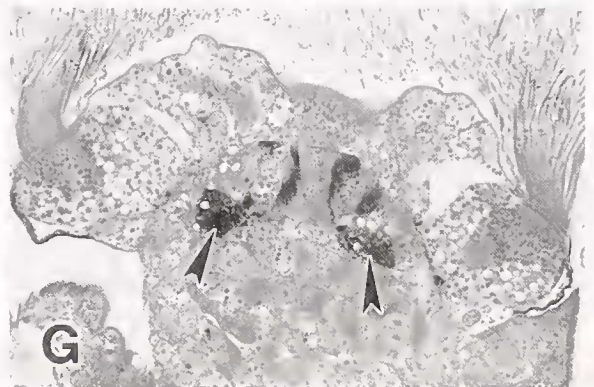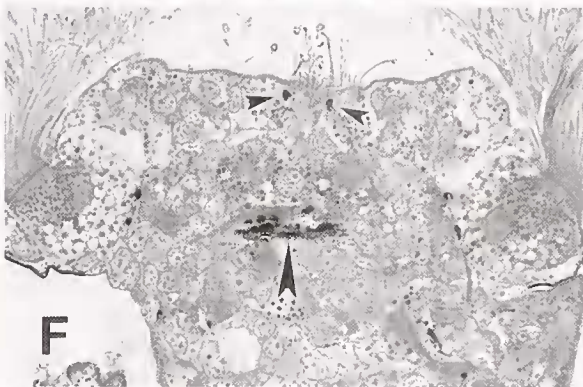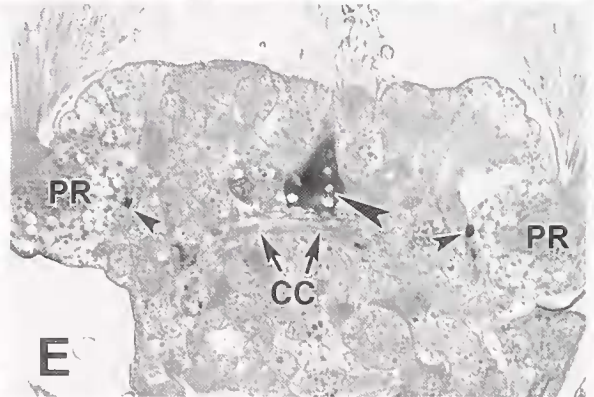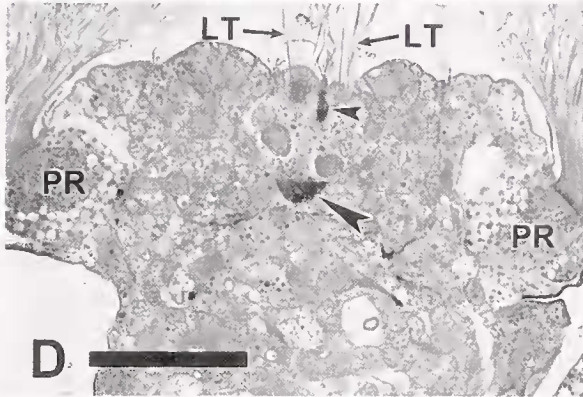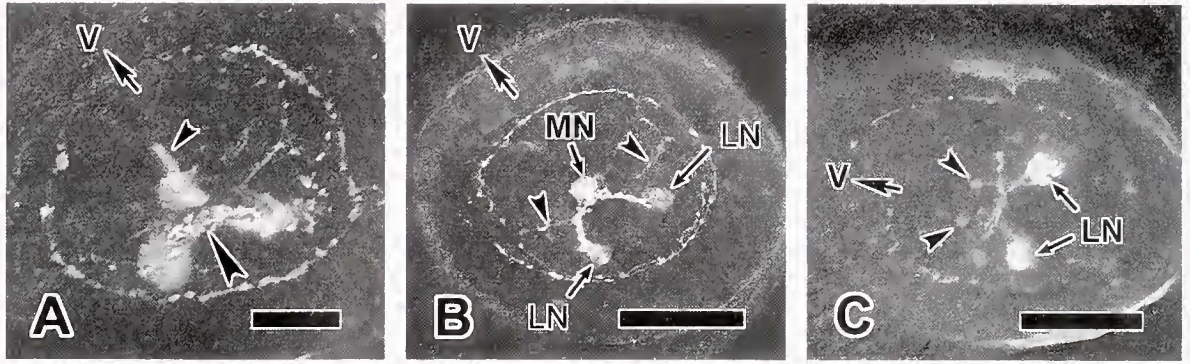
this neuron corresponds to a medially located neuron expressing serotonin immunoreactivity and is therefore identified as the medial serotonergic neuron (MS) in Figure 5A, C. One of several cilia arising from this dendrite is shown in Figure 5B. The subepithelial cell bodies of the two sensory neurons of dendritic group 1 are sandwiched between the large, subepithelial cell bodies of the lateral tuft cells (Figs. 5C, 6A).

The largest number of dendrites within the larval apical ganglion of *T. scutum* are associated with the rim of the apical pit. Dendritic group 2 is located immediately ventral to the pit (Figs. 3A, 4), whereas groups 3 and 4 are located to the left and right, respectively, of the pit (Figs. 3A, 4, 6A, B). The apices of dendrites within groups 5 and 6 are located to either side of the ciliary tuft cells (Fig. 2A, B), but they deflect mediodorsally as they descend through the apical epidermis so as to join the ipsilateral group of dendrites on either side of the apical pit (Fig. 2A, B).

Neuropil of the apical ganglion

Neuronal cell bodies of the apical ganglion overlie a tangled collection of interdigitating neurites. Neurites of this ganglionic neuropil are characterized by large swellings stuffed with electron-dense vesicles (Fig. 6C). The neuropil overlies the cerebral commissure, a tract of neurites connecting between the two developing cerebral ganglia (Fig. 6A), but two stubby horns of the neuropil project dorsolaterally. The neuropil is ultrastructurally distinct from the cerebral commissure because neurites of the commissure tend to have parallel trajectories as they extend between the two cerebral ganglia and commissural neurites lack large swellings filled with masses of electron-dense vesicles.

Serotonin immunoreactivity within the apical ganglion and peripheral neurites

Three apical neurons, a medial neuron located centrally beneath the apical disc and two neurons located dorsolateral to the medial neuron, are recognized by antibodies against serotonin in veligers fixed at 52 h and 90 h postfertilization (Fig. 7A, B), and at 5 days postfertilization. A neurite from each lateral neuron extends ventrally to a concentration of neurites located beneath the medial, serotonin-immunoreactive neuron. The fluorescent label identifies large varicosities within the central concentration of neurites (Fig. 7A). Peripheral neurites extend laterally from each side of the central concentration of neurites and continue along the base of the large, prototrochal ciliated cells that encircle the apical disc (Fig. 7A, B). These peripheral neurites have a beaded appearance.

Immunolabeled larvae that were sectioned after processing for diaminobenzidine (DAB) reaction product show that the medial serotonin-immunoreactive neuron is the non-ampullary sensory neuron that contributes one of the two dendrites belonging to dendritic group 1 (Fig. 7D, E, H). The lateral neurons have dendrites belonging to dendritic groups 3 and 4, which lie on either side of the apical pit (Fig. 7F, I). Although the stout dendrite of the medial neuron can often be seen in fluorescently labeled larvae (Fig. 7B), the slender dendrites of the lateral neurons are difficult to ascertain without sections prepared from DAB-

Figure 7. Serotonin immunoreactivity within apical ganglion neurons and peripheral neurites of *Tectura scutum* veligers. (A-C) Confocal scanning laser micrographs of fluorescently labeled whole larvae in apical view; arrows labeled "V" indicate ventral side. (A) Larva at 90 h postfertilization (velar margin slightly contracted) showing label within the neuropil (large arrowhead) and the stout dendrite (small arrowhead) arising from the medial serotonergic neuron. Scale, 20 μm. (B) Larva at 90 h postfertilization showing label within a medial and two lateral neurons, and peripheral tracts on either side (arrowheads) projecting to a circular tract running beneath the prototrochal cells. Scale, 40 μm. (C) Apical view of a larva at 7 days postfertilization showing label within the two lateral serotonergic neurons but not in the medial neuron; arrowheads indicate small labeled neurons within the developing cerebral ganglia. Scale, 40 μm. (D-G) Frontal sections (1-μm thickness) through the head of a *T. scutum* larva at 90 h postfertilization labeled with DAB reaction product (dark deposit in cells). Sections proceed from ventral to dorsal side of the apical sensory organ. Scale, 30 um. (D) Section passing through the ventral margin of the apical sensory organ showing the lateral ciliary tufts and the distal dendrite (small arrowhead) and portion of the basal soma (large arrowhead) of the medial serotonergic neuron. (E) Soma of the medial serotonergic neuron (large arrowhead); also note label within neurites at the base of the large prototrochal cells (small arrowheads) and absence of label within the cerebral commissure. (F) Label within the neuropil (large arrowhead) of the apical ganglion; also note label within distal dendrites (small arrowheads) of two lateral serotonergic neurons. (G). Section through the somata of two lateral serotonergic neurons showing their irregular shape. (H-I) Transmission electron micrographs of sections cut parallel to the apical surface of a DAB-labeled *T. scutum* larva at 52 h postfertilization. (H) Dendritic profiles of the ampullary neuron and non-ampullary neuron (arrow) within dendritic group 1; DAB label within the latter profile identifies it as the medial serotonergic neuron. Scale, 2 μm. (I) Dendritic profiles (asterisks) located to the immediate left of the apical pit; the most peripheral profile contains DAB label (large arrow). Scale, 2 μm. Abbreviations: AM, ampullary neuron; AP, apical pit; CC, cerebral commissure; LN, lateral serotonergic neuron; LT, lateral ciliary tuft; MN, medial serotonergic neuron; PR, prototrochal cell.

labeled specimens. Sections of DAB-labeled specimens also reveal that the cell bodies of the lateral neurons have a very irregular outline (Fig. 7G). The central concentration of varicose neurites identified in fluorescent images corresponds to the neuropil of the apical ganglion (Fig. 7F).

In metamorphically competent larvae (7 days postfertilization), antibodies against serotonin continue to label the two lateral neurons and the peripheral ring of neurites underlying the prototrochal ciliated cells, but a medial neuron does not label (Fig. 7C). However, smaller neurons within the cerebral ganglia begin to express serotonin antigenicity at 7 days postfertilization (Fig. 7C). Despite loss of serotonin antigenicity within the medial neuron in late-stage larvae, this neuron can still be identified in sections of 7-day larvae examined by transmission electron microscopy (Fig. 8). Positive identification of this neuron is facilitated by the fact that its dendrite has a stereotypical location relative to that of the distinctive ampullary neuron and the lateral ciliary tuft cells (Fig. 2B).

Discussion

A goal for this study was to compare the apical sensory organ in a patellogastropod veliger to that of apogastropod veligers (caenogastropods and heterobranchs) in order to identify conserved and variant aspects of this larval neural structure among gastropod molluscs. However, uncertainty about the cellular constituents that are properly included within the structural entity that has been called the "apical organ," the "apical sensory organ," and the "cephalic sensory organ" (see Kempf *et al.*, 1997) can complicate comparisons among larval gastropods and among larval spiralians in general. As previously stated, I use the term "apical sensory organ" to refer to the cellular complex that includes both the apical ganglion and cells bearing prominent ciliary structures at the larval apex. It is unclear if, among gastropod veligers, the distinctive apical ciliated cells are a type of sensory neuron (Chia and Koss, 1984; Marois and Carew, 1997a; Kempf *et al.*, 1997; Page and Parries, 2000). Additional work is needed to resolve this issue.

A second complicating factor for comparisons of the apical sensory organ among gastropod larvae derives from differences in life history. For most patellogastropods, fertilization is external, and a trochophore-like larvae hatches from the egg investments after prototrochal cilia have differentiated but before most other components of the veliger larval form have developed (Smith, 1935; Wanninger *et al.*, 1999, 2000; present study). The shell, foot, retractor muscles, and gut emerge rapidly over the course of a relatively short, nonfeeding larval phase. By contrast, the ancestral pattern for the Apogastropoda, which is retained by many extant species, involves internal fertilization and deposition of encapsulated eggs that eventually hatch as feeding veliger larvae with well-formed shell, foot, larval retractor

muscle, and gut. After hatching, planktotrophic apogastropod veligers typically undergo further growth and development during a planktonic life lasting weeks or months before metamorphic competence is achieved. Life-history differences between patellogastropods and apogastropods mean that differences in apical organ structure between a single developmental stage from each group may be merely maturational differences in a common developmental program for both.

In an effort to distinguish truly novel features for the apical sensory organ in veliger larvae of *T. scutum* together with features shared with planktotrophic apogastropod veligers, as described in greatest detail by Chia and Koss (1984), Kempf *et al.* (1997), Marois and Carew (1997a), and Page and Parries (2000), I examined a range of larval stages for this patellogastropod. The similarities include a bilateral pair of lateral ciliary tuft cells, neurons clustered over a neuropil to form a simple ganglion, a tripartite distribution of neuronal dendrites extending from the ganglion to the apical surface, and a tripartite distribution of serotonin-immunoreactive neurons with peripheral neurites extending to the base of the ciliated prototrochal cells. Features of the apical sensory organ of *T. scutum* that have not been previously identified among apogastropod veligers include a centrally located tuft of very long apical cilia that is lost early in larval development, the presence of only a single ampullary neuron, and the loss of serotonin immunoreactivity by the medial serotonergic neuron during late larval development. These comparisons are discussed further below.

Apical and lateral ciliary tufts: identifying homologs in other gastropods

Apical ciliary structures in veliger larvae of apogastropods have been difficult to reconcile with the condition in larvae of patellogastropods and of many other molluscan classes, where a long apical tuft of nonmotile cilia is produced by some or all of the apical rosette micromeres of the embryo (see Raven, 1966; van Dongen and Geilenkirchen, 1974; Verdonk and van den Biggelaar, 1983; Dohmen, 1992; Dictus and Damen. 1997). Observations on multiple larval stages of *T. scutum* help to resolve this uncertainty because larvae of this species have both a centrally located, very long apical ciliary tuft as well as two lateral tufts that flank the midline and consist of shorter cilia.

The lateral ciliary tuft cells in *T. scutum* are strikingly similar in both position and morphology to the bilateral multiciliated cells reported previously for planktotrophic opisthobranch larvae (Heterobranchia) (Chia and Koss, 1984; Kempf *et al.*, 1997; Marois and Carew, 1997a). In both groups, the cells have expanded, multiciliated apices and a narrow, stalk-like middle zone that connects with a basal, perinuclear region. The perinuclear regions of the
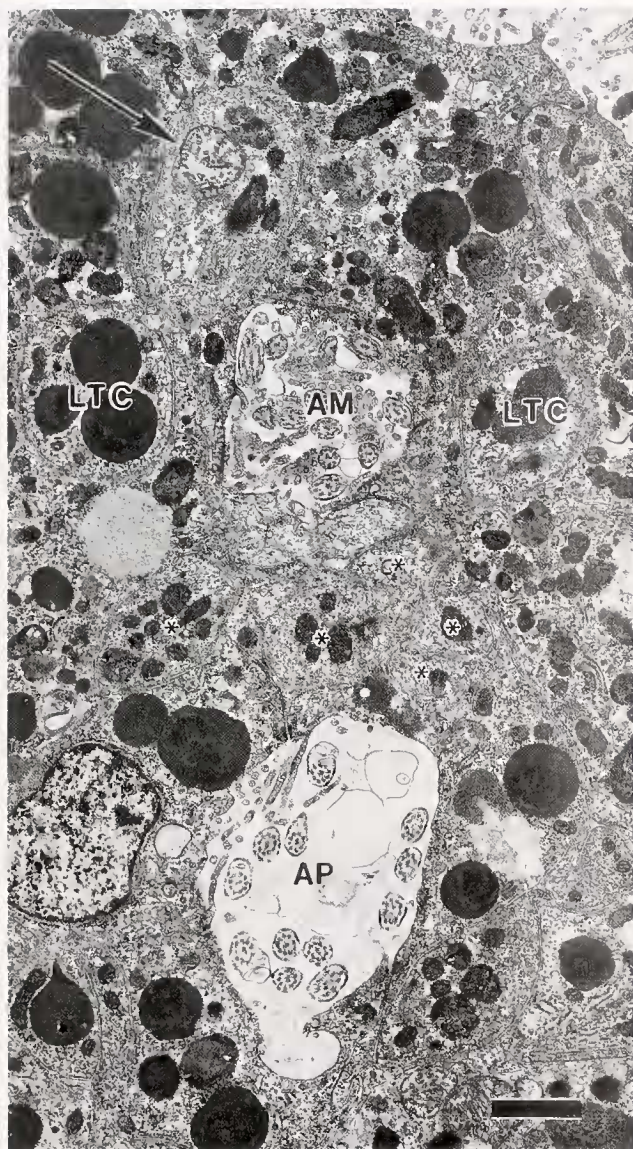
Figure 8. Transmission electron micrograph of a section cut parallel to the apical surface of a *Tectura scutum* larvae at 7 days postfertilization (metamorphic competence). Large arrow indicates dendrite of medial sensory neuron, which persists despite loss of serotonin immunoreactivity at this stage. Asterisks indicate dendrites within dendritic group 2. Scale, 1 μm. Abbreviations: AM, ampullary neuron; AP, apical pit; LTC, lateral ciliary tuft cell.

lateral ciliary tuft cells are intimately associated with neuronal somata along the ventral side of the apical ganglion. Furthermore, the expanded apices of the lateral ciliary tuft cells flank the dendrites of the medial serotonergic neuron and the medial ampullary neuron in planktotrophic veligers of several nudibranch larvae (Kempf *et al.*, 1997) and in veligers of *T. scutum* after disappearance of the three large cells that generate the long, centrally located apical ciliary tuft (Figs. 2B, 4, 5A).

The lateral ciliary tuft cells within the apical epidermis of

caenogastropod larvae show fewer similarities to those of *T. scutum* and opisthobranch larvae. In two caenogastropods where lateral ciliary tufts have been identified (*Lacuna vincta* and *Euspira lewisii*; Page and Parries, 2000), the multiciliated cells have a cuboidal shape and are entirely contained within the thickness of the apical epidermis (Page and Parries, 2000). These multiciliated cells lie ventral to the apical ganglion, but they are not intimately associated with any of the neuronal elements of the ganglion.

In Smith's (1935) description of larvae belonging to the patellogastropod *Patella vulgata*, he used the term "refractile bodies" for what are probably the protruding, multiciliated apices of lateral ciliary tuft cells. However, contrary to observations reported here for *T. scutum*, Smith (1935) suggested that the centrally located, long apical tuft of *P. vulgata* (another patellogastropod) persists up to metamorphosis, whereas the flanking ciliated cells (refractile bodies) degenerate early in larval development. This discrepancy between reports on *T. scutum* and *P. vulgata* may represent a real difference between larvae of these two patellogastropods. However, Smith (1935) was restricted to observations using light microscopy; his observations on the fate of apical ciliary structures in *P. vulgata* larvae should be confirmed using electron microscopical techniques.

Larvae of many bivalve species possess a single long apical ciliary tuft that is often lost during later development (Cragg, 1996). Ultrastructural observations by Tardy and Dongard (1993) show that the apical ciliary tuft ("frontal cirri") of the bivalve *Ruditapes philippinarum*, like that of *T. scutum*, is generated by three large apical cells containing very long ciliary rootlets. The apical tuft of *R. philippinarum* persists until metamorphosis.

Results of the present study on *T. scutum*, when combined with previous observations on the apical sensory organ of other gastropods and other molluscs, suggest that a single, centrally placed tuft of very long apical cilia and a bilateral pair of shorter ciliary tufts are both plesiomorphic traits of the apical sensory organ among gastropod veligers. As discussed above, veligers of both basal and derived clades of gastropods appear to possess a bilateral pair of short ciliary tufts. Furthermore, larvae of the most basal clade of gastropods, the Patellogastropoda, together with larvae of many nongastropod molluscs, have a centrally placed tuft of long apical cilia that arises from descendants of the apical rosette micromeres. Under this hypothesis, evolutionary descendants of the apical rosette micromeres in posthatching apogastropod veligers have lost the long apical tuft phenotype. Homologous cells in developing apogastropods may degenerate prior to hatching or may have been co-opted in prehatching stages to serve a derived function associated with encapsulation. Many caenogastropod embryos develop a crown of motile cilia that is lost shortly before or after hatching, and descendants of the apical rosette micromeres may contribute to this ciliary

crown (Conklin, 1897; Cather, 1973). Beating of these crown cilia may supplement the beating of prototrochal cilia to help prevent buildup of an anoxic boundary layer around closely packed embryos (Hunter and Vogel, 1986; alternative view given by Strathmann and Strathmann, 1995). This hypothesis about derived states of the apical tuft cells in apogastropod embryos should be explored using cell lineage tracers in conjunction with ultrastructural analysis.

Larval apical ganglion of T. scutum (Patellogastropoda): comparisons to apogastropods

Existing information suggests that the apical ganglion of planktotrophic larvae of caenogastropods and opisthobranch members of the Heterobranchia includes neurons with and without an apical dendrite and that the ganglion provides innervation of prototrochal cilia and muscles of the larval velum (Kempf et al., 1997; Marois and Carew, 1997a, c; Page and Parries, 2000; Hadfield et al., 2000; Hay-Schmidt, 2000). Neuronal elements of the apical sensory organ of T. scutum are also organized as a simple ganglion, with neuronal somata overlying a neuropil. Many neurons of the apical ganglion in T. scutum have a distal dendrite and are presumably sensory, but the possible presence of neurons lacking an apical dendrite was not ascertained for T. scutum because neurons without an apical dendrite were not recognized by antibodies against serotonin.

Ampullary neurons represent a morphologically distinctive class of sensory neurons within the apical ganglion of apogastropod larvae and have also been reported in a larval bivalve (Tardy and Dongard, 1993). However, apogastropods have a minimum of four ampullary neurons within the larval apical ganglion (see Chia and Koss, 1984; Marois and Carew, 1997a, c; Kempf et al., 1997; Page and Parries, 2000), whereas larvae of T. scutum have only one neuron of this distinctive type. Cilia within the invaginated pocket of ampullary neurons have a $9 + 2$ arrangement of axonemal microtubules in veligers of T. scutum (present study), which is also true of larval opisthobranchs (Bonar, 1978; Chia and Koss, 1984; Kempf et al., 1997; Marois and Carew, 1997a) and larvae of the bivalve Rhuditapes philippinarum (Tardy and Dongard, 1993). The ciliary axonemes of ampullary neurons in caenogastropods show differences from the $9+2$ pattern (Uthe, 1995; Page and Parries, 2000).

Dendrites arising from sensory neurons of the apical ganglion in planktotrophic larvae of caenogastropods and opisthobranch heterobranchs are organized into three bundles: a medial bundle and two lateral bundles (Chia and Koss, 1984; Kempf et al., 1997; Marois and Carew, 1997a). This tripartite organization is not readily apparent in pretorsional veligers of T. scutum. However, after the three large cells that produce the apical ciliary tuft disappear, the dendritic components in the apical epidermis shift centrally and a three-part disposition of dendrites becomes easier to recognize, when compared to the condition in posthatching opisthobranch veligers. I suggest that dendritic groups 1 and 2 in T. scutum, which collectively include the ampullary neuron and medial serotonergic neuron, correspond to the medial dendritic grouping in opisthobranch veligers, which also includes a single ampullary and serotonergic sensory neuron (Kempf et al., 1997; Marois and Carew, 1997a). I further suggest that the dendritic groups 3 and 5 on the left side of the apical pit and groups 4 and 6 on the right side of the apical pit correspond to the left and right lateral dendritic groups within the apical ganglion of opisthobranch veligers. In opisthobranchs (Aplysia californica and several nudibranchs) and in T. scutum, each lateral group of dendrites includes a single dendrite arising from a serotonergic sensory neuron.

Comparative patterns of serotonin immunoreactive neurons in larval gastropods

Among gastropod larvae studied to date, larvae of T. scutum are not unusual in having a tripartite distribution of neurons showing serotonin immunoreactivity (see Marois and Carew, 1997a, b, c; Kempf et al., 1997; Page and Parries, 2000). However, two aspects of the serotonin-immunoreactive neurons in T. scutum are unprecedented. First, all three serotonergic neurons within the apical ganglion of T. scutum have a distal cytoplasmic extension ("dendrite") that runs to the external surface of the apical epidermis. Therefore, these are all putative sensory neurons. In apogastropod veligers studied to date, at least some of the serotonin-immunoreactive neurons within the apical ganglion lack distal dendrites. Second, one of the three serotonergic neurons in T. scutum (the non-ampullary neuron within dendritic group 1) appears to arrest expression of serotonin in metamorphically competent larvae. Nevertheless, the possibility that the medial serotonergic neuron is lost entirely and replaced by a new neuron that lacks serotonin antigenicity cannot be ruled out.

Assuming that neurites of the medial neuron also lose serotonin antigenicity in metamorphically competent larvae (or these neurites are lost entirely), persistence of serotonin immunoreactivity within neurites along the base of the prototrochal ciliary cells implies that the prototrochal neurites originate from the lateral serotonergic neurons of the apical ganglion. If this is the case, then the lateral neurons may function as both sensory and motor neurons. Hay-Schmidt (2000) has speculated that lateral serotonin-immunoreactive neurons within the apical ganglion of diverse spiralian larvae always innervate the larval ciliary band.

A similar case of developmental arrest of neurotransmitter synthesis without destruction of the neuron has been reported by Diefenbach et al. (1998) for a pair of apically located sensory neurons (ENC1) within encapsulated embryos of the pulmonate Helisoma trivolvis (Hetero-

branchia). This species lacks a free-living larval stage, and development is highly modified relative to heterobranchs with a planktotrophic larva. Early expression of serotonin immunoreactivity by the ENC1 neuronal pair is lost during later embryogenesis.

Transient expression of serotonin by an apical ganglion neuron in *T. scutum* may occur because of ontogenetic change in a larval behavior, possibly associated with settlement and metamorphosis, which removes the functional requirement for this neurotransmitter. Hadfield *et al.* (2000) have recently described evidence for apical ganglion involvement in reception of the metamorphic cue in an opisthobranch. However, a second possibility relates to the established role of serotonin in regulating aspects of molluscan neurogenesis (Haydon *et al.*, 1984, 1987; Murrain *et al.*, 1990; Zhu *et al.*, 1994; Diefenbach *et al.*, 1995). Temporary developmental expression of serotonin by an apical ganglion neuron might reflect a transient role for this transmitter in establishing neurite trajectories or neurite branching patterns within the larval or prospective, postmetamorphic nervous system. The notion that neurites extending from the apical ganglion may help organize later formed components of the nervous system has been suggested previously by Lacalli (1981, 1984), on the basis of studies of several species of polychaete trochophores.

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